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                 D TI L1 1-30
                 D BIB AB 21
                 D BIB AB L1 23 21 22
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                 D TI 1-57
                 D BIB AB 32 41 27
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Examiner Search Notes

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Effect of Heme Oxygenase Inhibitors on Soluble Guanylyl Cyclase Activity

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NO is the physiological activator of soluble guanylyl cyclase (sGC) thereby acting as a signaling molecule in the nervous and cardiovascular systems. Despite its poor sGC-activating ability, CO, produced by the enzyme heme oxygenase (HO), has also been implicated as a physiological stimulator of sGC in neurotransmission and vasorelaxation. Zinc protoporphyrin IX (ZnPPIX) and tin protoporphyrin IX (SnPPIX) are competitive HO inhibitors and have been used in studies implicating a messenger role for CO in the brain and periphery; however, little is known about the specificity of these metalloporphyrins. In the present study, the effects of ZnPPIX and SnPPIX on sGC activity have been investigated *in vitro*. Interestingly, purified sGC is markedly activated by SnPPIX (20- to 30-fold) but has a very low affinity for this metalloporphyrin (K_a = 4.9 μM); high concentrations of SnPPIX (25 μM) still activated the enzyme. On the other hand, sGC has a high affinity for ZnPPIX (K_a = 16.1 nM). ZnPPIX activates heme-containing sGC weakly at low (nM) concentrations (3- to 4-fold) but at higher concentrations, ZnPPIX is a potent inhibitor; at 2.5 μM, it inhibits the basal activity of sGC by about 80%. These results imply that HO inhibitors may affect cGMP levels independently of HO activity. © 1998 Academic Press

Key Words: **soluble guanylyl cyclase; heme oxygenase; carbon monoxide; SnPPIX; ZnPPIX.**

Soluble guanylyl cyclase (sGC)[2] [EC 4.6.1.2] catalyzes the formation of cGMP from GTP and represents the major target for nitric oxide (NO) (1). NO is synthesized from L-arginine by a family of NO synthases (NOS) [EC 1.14.13.39] (2, 3) and stimulates sGC several hundredfold (4–6). This activation results in a pronounced accumulation of cGMP, an important second messenger involved in a variety of physiological processes such as smooth muscle relaxation (7–9), platelet aggregation (10–12), inflammation (13, 14), and neurotransmission (3, 15). sGC is a cytosolic heterodimer of 150 kDa, with subunits of similar sizes (16, 17). Catalytic activity requires the presence of either Mg^{2+} (the natural cofactor) or Mn^{2+} in excess of the substrate (18). sGC contains a noncovalently bound prosthetic heme which is necessary for NO-induced stimulation but is not essential for activity (4, 19). Spectral studies of sGC reveal a five- or six-coordinate heme with histidine(s) as the axial ligand(s) (5, 20–22). NO binds with high affinity to the prosthetic heme group (23) causing the scission of the proximal His–Fe bond and the formation of a five-coordinate ferrous–nitrosyl complex (20, 22, 24, 25). It is proposed that a subsequent conformational change of the enzyme results in an up to 400-fold increase in cGMP production (5, 6). In support of this hypothesis, protoporphyrin IX, the metal-free cofactor which cannot form an axial metal–ligand bond, activates the enzyme independently of NO (18, 26).

Carbon monoxide (CO) has also been implicated as a physiological stimulator of sGC (27, 28). CO is pro-

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[2] Abbreviations used: BSA, bovine serum albumin; CO, carbon monoxide; CoPPIX, cobalt(II) protoporphyrin IX; cGMP, guanosine-3′,5′-cyclic monophosphate; DMSO, dimethyl sulfoxide; DTT, D,L-dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FePPIX (heme), iron(II) protoporphyrin IX; FePPIX(NO), iron(II) protoporphyrin IX-nitrosyl; GTP, guanosine-5′-triphosphate; HO, heme oxygenase; IBMX, 1-methyl-3-isobutylxanthine; βME, β-mercaptoethanol; MnPPIX, manganese(II) protoporphyrin IX; NO, nitric oxide; PEI-F, polyethyleneimine cellulose with fluorescent indicator; SDS, sodium dodecyl sulfate; sGC, soluble guanylyl cyclase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SnPPIX, tin(IV) protoporphyrin IX (2+); sGC, soluble guanylyl cyclase; TEA, triethanolamine HCl; TLC, thin-layer chromatography; Tween 20, poly(oxyethylene)$_{20}$-sorbitane monolaurate; ZnPPIX, zinc(II) protoporphyrin IX.

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duced by the enzyme heme oxygenase (HO) [EC 1.14.99.3] which converts heme to biliverdin and CO. HO exists as two isoforms, of which type 1 (HO-1) is inducible and type 2 (HO-2) is constitutively expressed (29, 30). HO-1 is highly expressed in liver and spleen where it catabolizes heme from hemoglobin (29) and HO-2 is widely expressed with high concentrations in the brain (31, 32). Interestingly, HO-2 was shown by *in situ* hybridization to colocalize with sGC in the brain (31, 32), suggesting a role for CO in neurotransmission through the cGMP pathway (32, 33). Metabolic labeling experiments permitted the direct measurement of ^{14}CO production by neurons *in vitro* and showed that CO release parallels endogenous cGMP concentrations (34). HO-1 and HO-2 were also detected in endothelial cells (35, 36), thus possibly being involved in vasorelaxation induced by CO (37–39). Exogenously applied CO has been shown to relax various isolated blood vessels and to increase cGMP levels (28). CO has also been implicated as a biological signal in the immune system (40) and in inhibition of the aggregation of human platelets (27).

Thus CO exhibits similar physiological properties to NO and it is believed that these functions are mediated by the ability of CO to act as an activator of sGC, resulting in elevated cGMP levels. CO, like NO, binds to the heme in sGC with high affinity but only leads to a two- to six-fold activation of the purified enzyme (5, 21, 41–43). In contrast to NO, CO forms a six-coordinate heme complex, with the proximal His–Fe bond remaining intact (5, 20, 22, 44). It has been suggested that dissociation of CO proceeds via a five-coordinate intermediate (42) which, by its structural similarity to the nitrosyl–heme complex, presumably is responsible for the observed minor stimulation of sGC by CO. Comparing the extent of activation induced by NO and CO, it remains unclear how CO can exert the physiological functions attributed to it because it is such a poor activator of sGC. One explanation has been suggested in a recent study showing that the xenobiotic YC-1 potentiated the effect of CO as an activator of sGC. In fact, a combination of CO and YC-1 caused greater sGC activation than that observed with NO alone (43, 45). Thus, the existence of an endogenous YC-1-like substance would provide molecular basis for a physiological role for CO in the regulation of sGC.

ZnPPIX and SnPPIX have been widely used to infer a role for HO-derived CO in signal transduction via cGMP (32, 34–36, 38), despite a complete lack of knowledge of the direct effect of these metalloporphyrins on the activity of the cGMP-generative enzyme, sGC. ZnPPIX and SnPPIX are competitive HO inhibitors (46) and SnPPIX has been used clinically for the inhibition of HO activity to prevent neonatal jaundice (47). However, the selectivity of ZnPPIX and SnPPIX is still controversial (35, 36, 48, 49) and concern has been raised that these porphyrins may have effects on other hemoproteins (50, 51). It has been shown in several studies that ZnPPIX and SnPPIX have a substantial effect on sGC and NOS activity in vascular and neuronal tissues (35, 36, 48, 49). In the present study we investigated the effects of the HO inhibitors SnPPIX and ZnPPIX on purified sGC activity *in vitro* and compared them to those observed with PPIX. Our data demonstrate that SnPPIX activates sGC in the micromolar concentration range to the same extent as PPIX and that ZnPPIX has a slight stimulatory effect in the nanomolar range but inhibits sGC in the micromolar range. Thus, inferences of the effect of CO that are made based on experiments using HO inhibitors may be of questionable validity given the varying effects of HO inhibitors on sGC activity. Some of the pharmacological effects observed with these drugs may derive from their direct effect on cGMP formation, independent of CO.

EXPERIMENTAL PROCEDURES

Materials

Bovine lung was obtained from the first cow slaughtered that day at Schroedl's Meat Market (Jefferson, WI). LiCl was obtained from Aldrich. NaCl was purchased from Fisher Scientific. ATP-agarose (9-atom spacer at C8), benzamidine, bovine serum albumin (BSA), D,L-dithiothreitol (DTT), EDTA, γ-globulin, cGMP, GTP, GTP-agarose (11-atom spacer at ribose hydroxyl), hemin chloride, 1-methyl-3-isobutylxanthine (IBMX), leupeptin, triethanolamine HCl (TEA), and trypsin inhibitor were all purchased from Sigma. [α-^{32}P]GTP was purchased from NEN–DuPont and EM Science PEI-F cellulose TLC plates (Merck) were obtained from VWR. Q Sepharose Fast Flow and NICK desalting columns were obtained from Pharmacia. Ultrogel AcA 34 resin was obtained from BioSepra. Tween 20 was purchased from Bio-Rad. Microbicinchoninic acid (μBCA) assay reagents were obtained from Pierce and SnPPIX, PPIX, and ZnPPIX were from Porphyrin Products. $MgCl_2.6H_2O$ was obtained from Mallinckrodt. Biosafe II scintillation cocktail was obtained from Research Products International. SNAP was a gift from Dr. J. Fukuto, UCLA School of Medicine.

Methods

Purification of soluble guanylyl cyclase from bovine lung. The enzyme used in the experiment summarized in Fig. 3 was isolated using a previously reported procedure (25). In all other experiments the enzyme used was purified through the following modified preparation. One kilogram of diced fresh bovine lung was washed with 25 mM TEA buffer, pH 7.8, and minced in a food processor. After being washed with 1 mM EDTA, 25 mM TEA buffer, pH 7.8, and then with 25 mM TEA, 50 mM βME buffer, pH 7.8, the tissue was diluted to 40% (w/v) with 25 mM TEA, 50 mM βME, 2 mM benzamidine buffer, pH 7.8. The minced lung tissue was then homogenized in a Waring blender. The homogenate was centrifuged at 17,000*g* for 30 min and the supernatant was brought to 50 mM NaCl and batch-adsorbed onto 650 mL of Q Sepharose Fast Flow ion exchange resin for 90 min with TEA, βME buffer, pH 7.8 (25 mM TEA, 50 mM βME, 0.2 mM benzamidine, 0.5 mg/mL trypsin inhibitor, 0.5 mg/mL leupeptin, 50 mM NaCl). The batch-adsorbed resin was vacuum filtered on a Büchner funnel, washed with TEA, βME buffer, pH 7.8, until the effluent was clear, and then equilibrated with TEA, DTT buffer, pH

7.8 (25 mM TEA, 5 mM DTT, 0.2 mM benzamidine, 0.5 mg/mL trypsin inhibitor, 0.5 mg/mL leupeptin, and 50 mM NaCl). The resin was then poured into a column (5 cm diameter), packed, and washed in the same buffer at 7 mL/min until the A_{280} of the effluent reached baseline. The protein was eluted with a 1.5-L linear NaCl gradient ranging from 50 to 700 mM NaCl in TEA, DTT buffer, pH 7.8. The active fractions (65% of maximal activity) were pooled, dialyzed overnight against 12 L of TEA, DTT buffer, pH 7.4, containing 4 mM $MgCl_2$, and centrifuged for 30 min at 17,000*g*. The supernatant was batch-adsorbed for 90 min onto 100 mL of ATP-agarose which had previously been equilibrated in TEA, DTT buffer, pH 7.4, containing 4 mM $MgCl_2$. The resin was then poured into a column (2.5 cm diameter), packed, and washed in the same buffer at 2.5 mL/min until the A_{280} of the effluent reached baseline. The protein was eluted with a 400-mL linear NaCl gradient ranging from 50 to 700 mM NaCl in TEA, DTT buffer, pH 7.4. The active fractions (65% of maximal activity) were pooled and concentrated with an Amicon concentrator (PM 30) to 15 mL. The concentrated protein was then loaded onto an AcA 34 size exclusion resin (2.5 × 120 cm) previously equilibrated in TEA, DTT buffer, pH 7.4. The protein was then slowly eluted (0.33 mL/min) with 400 mL of the same buffer. The active fractions (65% of maximal activity) were then pooled and concentrated to 5–10 mL. The concentrated protein was brought to 4 mM $MgCl_2$ and slowly loaded onto a 5-mL GTP-agarose column. The sGC was allowed to bind to the column for 20 min and then washed with 15 mL of TEA, DTT buffer, pH 7.4, containing 4 mM $MgCl_2$. The enzyme was then eluted with 15 mL of 700 mM NaCl, TEA, DTT buffer, pH 7.4. The active fractions were pooled and concentrated to approximately 1 mL in Amicon Centricon 30 microconcentrators. The purified enzyme was then stored in 20- or 50-μL aliquots at −80°C. The two different batches of enzyme used were tested for their BSA content by Western blot analysis and did not show any trace of BSA. Their specific activities ranged in between 20 and 200 nmol/min/mg and the activation observed with SNAP ranged in between 40- and 130-fold over the basal activity.

Soluble guanylyl cyclase activity assay. The activity of sGC was determined as previously reported (52, 53) by the measurement of the formation of [^{32}P]cGMP from [α-^{32}P]GTP. Each enzyme reaction was conducted in a total volume of 200 μL containing 40 mM TEA, pH 7.4, 10 mM DTT, 1 mM GTP, 3 mM $MgCl_2$, 0.3 mM IBMX, 2.5 pmol [α-^{32}P]GTP, and variable amounts of sGC (0.3–1.5 μg), depending on the experiment. The reaction was carried out for 10 min at 37°C and stopped by adding 10 μL of 0.5 M EDTA and placement on ice. SNAP (100 μM) was used as an NO donor. The cGMP formed was separated from GTP by TLC on PEI-F cellulose plates using 0.15 M LiCl as the eluent. The cGMP was eluted into 10 mL of Biosafe cocktail and the radioactivity was determined on a Beckman LS-6000 scintillation counter. Protein concentrations were determined by the BCA or μBCA assay using γ-globulin as a standard. Samples containing DTT (5 mM) were read against a standard curve containing DTT (54). All data were obtained by taking the average value of two or three separate determinations; the data in the figures and tables are expressed as the mean plus or minus the deviation from the mean.

Heme depletion. The enzyme was heme depleted using a previously described method (55) modified as follows: the protein solution was gently mixed in a 1:1 volume ratio with a 1% Tween 20 solution in 25 mM TEA, pH 7.4, 5 mM DTT buffer (final concentration of Tween 20, 0.5%) for 5 min on ice and then loaded onto a NICK desalting column. The depleted enzyme was eluted in the void volume with 25 mM TEA, pH 7.4, 5 mM DTT buffer and used as is for further experiments. In the experiments in which heme-containing and heme-depleted sGC were compared for their response to exogenous porphyrins, the non-heme-depleted sGC was treated in the same way as described above, except that Tween 20 was omitted.

Porphyrin solution preparation. Stock solutions of PPIX, SnPPIX, and ZnPPIX were prepared freshly for each experiment by dissolving the porphyrin in DMSO and diluting it in 25 mM TEA buffer, pH 7.4. The final concentration of DMSO never exceeded 0.5% in the assay. We determined in a preliminary experiment that DMSO up to 10% (v/v) did not affect the activity or the NO activation of sGC.

Affinity and activation constant calculations. The K_a, K_m, and V_{max} values were calculated by nonlinear least-squares fit of the initial velocities to the Michaelis–Menten equation using the program HYPERO (56).

RESULTS

Effect of PPIX, SnPPIX, and ZnPPIX on sGC Activity

PPIX, SnPPIX, and ZnPPIX affect sGC activity; all three porphyrins activate sGC to varying extents before becoming inhibitors at higher concentrations. The concentration range in which activation was observed was different for each porphyrin, reflecting different affinities of sGC for PPIX, SnPPIX, and ZnPPIX (Fig. 1). PPIX significantly activated sGC over a concentration range from 10 nM to 1 μM; higher concentrations caused a decrease in the extent of activation and concentrations over 10 μM PPIX inhibited the basal activity of the enzyme (Fig. 1A). SnPPIX activated sGC over a concentration range of 1 to 20 μM (Fig. 1B). Concentrations in excess of 20 μM caused a decrease in the activation and very high concentrations (>100 μM) ultimately inhibited the basal activity of the enzyme (data not shown). ZnPPIX very weakly activated sGC over a narrow range of concentrations from 25 to 200 nM and concentrations above 250 nM inhibited the basal activity (Fig. 1C). Interestingly, in a similar experiment where the enzyme had a higher heme content, the concentration-dependence curve was shifted slightly and 1 μM ZnPPIX was still activating the protein (Table 2). In conclusion, all porphyrins tested alter sGC activity and all are inhibitory at high concentrations.

Magnitude of the Influence of PPIX, SnPPIX, and ZnPPIX on sGC Activity

At concentrations giving optimal response, PPIX, SnPPIX, and ZnPPIX show markedly different effects on enzyme activity. PPIX (100 nM) and SnPPIX (5 μM) significantly activate purified sGC, whereas ZnPPIX (200 nM) only stimulates the enzyme very slightly (Table 1). PPIX and SnPPIX activated sGC to a similar extent and the activation by the porphyrins was always less than that observed for NO; the enzyme was activated 22-fold by PPIX (100 nM) and 21-fold by SnPPIX (5 μM). In a similar experiment, with slightly higher porphyrin concentrations, 36-fold activation was observed with 200 nM PPIX and 24-fold activation with 15 μM SnPPIX (data not shown). In contrast, ZnPPIX (100 nM) caused only a 3-fold increase in the basal activity. Typically, when tested at concentrations

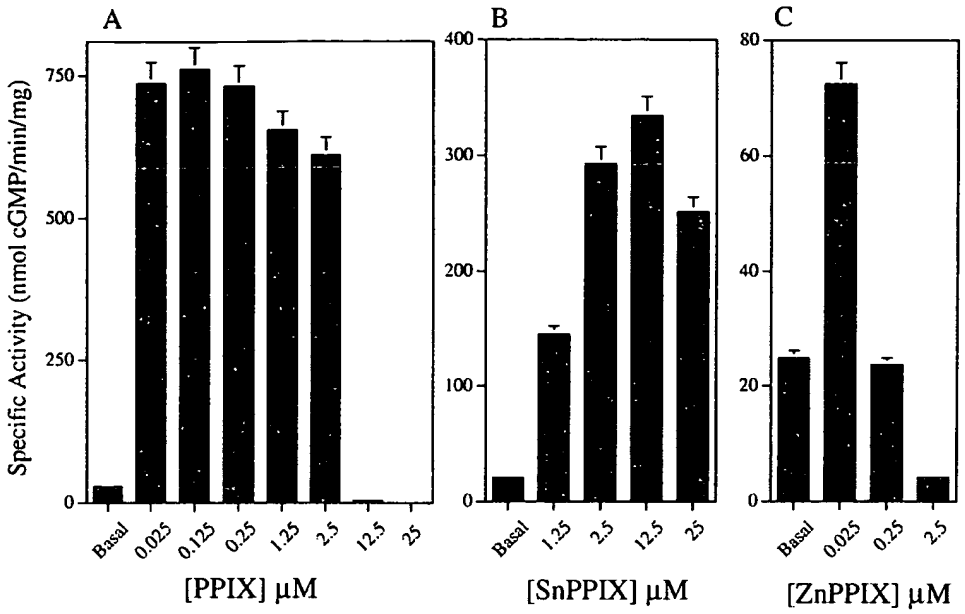

FIG. 1. Effect of PPIX (A), SnPPIX (B), and ZnPPIX (B) on sGC activity. The experiments were conducted with 10 min of preincubation of the porphyrin with the enzyme (0.74 μg) at 37°C prior to the assay. The volume of the preincubation mixture was 160 μL and the assay was begun by addition of the substrate solution. The porphyrin concentrations given are the final concentrations in the final 200-μL assay volume. The final concentration of sGC in the assay was 25 nM in experiments A and B and 50 nM in experiment C, respectively. Values represent means of two separate determinations; errors are the deviation from the mean.

where the maximum effect is seen, PPIX activates the enzyme more than does SnPPIX, which activates much more than does ZnPPIX. The activity measured in the presence of these porphyrins never reaches that observed in the presence of the NO-donor alone (Table 1).

Affinity of sGC for PPIX, SnPPIX, and ZnPPIX

sGC displayed different affinities for each of the porphyrins. sGC showed a high affinity for PPIX with an activation constant (K_a) for PPIX of 11.0 nM and a theoretical maximum velocity (V_{max}) of 7.4 μmol/min/mg (Fig. 2A). The activation constant obtained in this study is consistent with results previously published (18, 26). SnPPIX was able to activate sGC to the same extent as PPIX but sGC displayed low affinity for SnPPIX. The K_a for SnPPIX of 4.9 μM was three orders of magnitude larger than that for PPIX (Fig. 2B); nevertheless, the theoretical maximum velocity (6.4 μmol/min/mg) obtained was similar to that obtained for PPIX. ZnPPIX only slightly activated the enzyme, although sGC showed an affinity for ZnPPIX similar to that observed with PPIX. The K_a for ZnPPIX (16.1 nM) was in the same range as that obtained with PPIX, but the maximum velocity was much lower (0.4 μmol/min/mg) (Fig. 2C). The high affinity and poor activation observed are consistent with a previous study in which ZnPPIX was shown to be a competitive inhibitor of sGC activation by PPIX (K_i = 50 nM) (57).

Effect of PPIX and SnPPIX on the K_m of sGC for GTP

At a concentration giving the optimal response, PPIX and SnPPIX have little effect on the affinity of sGC for its substrate. Figure 3 illustrates Michaelis–Menten plots of velocity versus GTP concentration for sGC in the presence or the absence of PPIX (100 nM) or SnPPIX (20 μM). The activation of sGC by PPIX is

TABLE I

Comparative Activation of sGC in the Presence of PPIX, SnPPIX, and ZnPPIX

Porphyrin	Porphyrin concentration (μM)	Specific activity (μmol cGMP/min/mg)	Fold activation
None		0.110 ± 0.005	
None + SNAP		12.8 ± 1.8	116
PPIX	0.1	2.4 ± 0.7	22
SnPPIX	5	2.3 ± 0.7	21
ZnPPIX	0.2	0.37 ± 0.06	3

Note. The assay was conducted as described under Materials and Methods and was started with the addition of 0.3 μg of enzyme to a substrate solution containing the different porphyrins tested. The final protein concentration was 10 nM and the porphyrin concentrations given are the final concentrations in 200-μL assay volumes. The fold activation is the ratio of the specific activity in the presence of the porphyrin or SNAP (100 μM) to the basal activity of sGC. Values represent averages of three separate determinations; errors are the deviation from the mean.

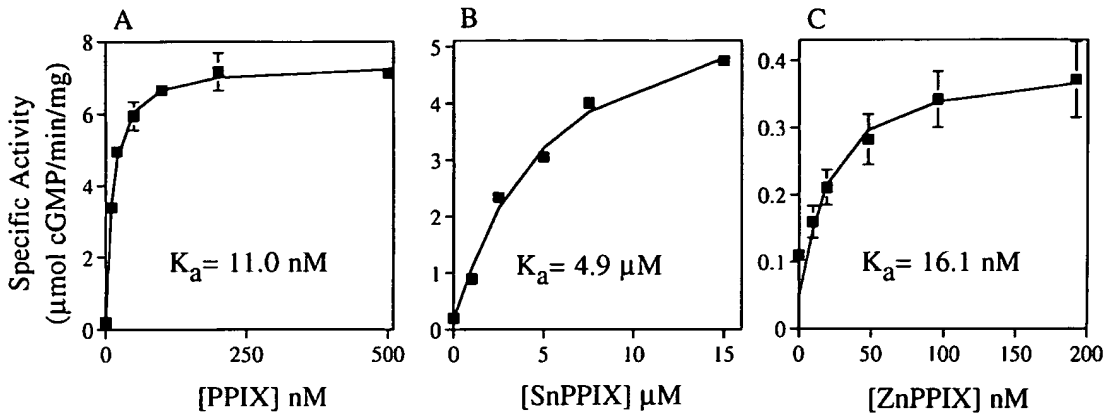

FIG. 2. Stimulation of sGC by PPIX (A), SnPPIX (B), and ZnPPIX (C). sGC activity was measured in the presence of increasing concentrations of PPIX (A), SnPPIX (B), and ZnPPIX (C). The final protein concentration in the assays was 10 nM (0.3 μg of enzyme was used per assay). Values represent averages of two (C) or three (A and B) separate determinations plus or minus the deviation from the mean. Where error bars do not appear, the standard deviation is smaller than the plotted point. The plotted points are measured specific activity values and the plotted lines are the calculated velocities at the same x values as calculated by HYPERO with linear interpolation between the points. The K_a values were obtained by nonlinear least-squares fit to the Michaelis–Menten equation (56) and were 11.0 ± 0.9 nM, 4.9 ± 0.9 μM, and 16.1 ± 1.9 nM for PPIX, SnPPIX, and ZnPPIX, respectively. The V_{max} values obtained were 7.4 ± 0.1, 6.4 ± 0.5, and 0.40 ± 0.01 μmol cGMP/min/mg for PPIX, SnPPIX, and ZnPPIX, respectively.

characterized by a marked increase in the V_{max} as well as a slight decrease in the K_m for GTP (113.2 μM vs 201.2 μM). These observations are consistent with those of Ignarro (26). Similarly, SnPPIX caused an equivalent increase in the V_{max}, and a slight decrease in the affinity of sGC for its substrate (151.4 μM vs 201.2 μM). The decrease in K_m observed upon activation of the enzyme by SnPPIX is less pronounced than that with PPIX, suggesting some difference between the activated conformations induced by the two porphyrins.

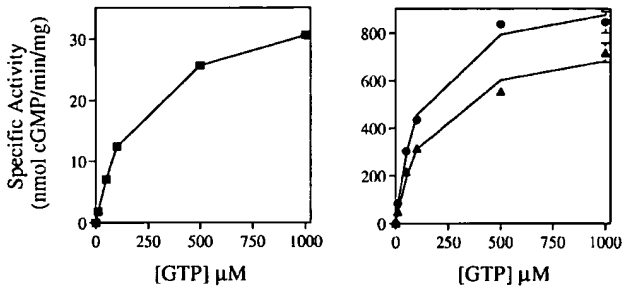

FIG. 3. Effect of PPIX and SnPPIX on the K_m for GTP and the V_{max} of sGC. Enzyme (1.46 μg) was incubated without (■) or with 100 nM PPIX (●) or 20 μM SnPPIX (▲). The final concentration of sGC in the assay was 50 nM and each assay was conducted in triplicate. The plotted points are measured specific activity values and the plotted lines are the calculated velocities at the same x values as calculated by HYPERO with linear interpolation between the points (56). The K_m values obtained using the HYPERO program were 201.2 ± 8.5, 151.4 ± 32.4, and 113.2 ± 15.9 μM without porphyrin, with SnPPIX, and with PPIX, respectively. The V_{max} values obtained were 36.5 ± 0.5, 784.7 ± 50.3, and 973.4 ± 38.6 nmol cGMP/min/mg without porphyrin, with SnPPIX, and with PPIX, respectively.

Affinity of Heme-Depleted sGC for PPIX, SnPPIX, and ZnPPIX

The effects observed with PPIX and SnPPIX on heme-depleted enzyme are similar to those observed with non-heme-depleted enzyme, whereas the observations with ZnPPIX differ (Table 2). When PPIX (200 nM) was incubated with sGC and heme-depleted sGC, the activation observed was 25- and 38-fold, respectively, showing a small increase in activation of the heme-depleted enzyme. Similarly, a separate experiment performed under the same conditions as the experiment described in Fig. 2 allowed us to determine the activation constant (K_a) for SnPPIX with non-heme-depleted and heme-depleted sGC (data not shown). The K_a and V_{max} obtained were 8.9 ± 4.2 μM and 1.11 ± 0.19 μmol cGMP/min/mg for non-heme-depleted sGC and 12.8 ± 1.4 μM and 1.03 ± 0.04 μmol cGMP/min/mg for heme-depleted SGC suggesting a similar effect in both cases. On the other hand, when 1 μM ZnPPIX was incubated with the non-heme-depleted sGC, the enzyme was activated 2.5-fold, whereas the heme-depleted cyclase was significantly inhibited at the same concentration.

DISCUSSION

There are numerous studies supporting the association between HO-derived CO and cGMP levels in the cell, and it is widely believed that CO exerts its effects on cGMP levels through the activation of sGC. For instance, HO-2 was shown by *in situ* hybridization to colocalize with sGC in numerous brain regions, positioning it appropriately to activate sGC (32, 34). Fur-

TABLE II

Effect of PPIX and ZnPPIX on Heme-Depleted sGC Activity

Treatment	Heme-containing sGC	Heme-depleted sGC
	Specific activity (μmol cGMP/min/mg)	
None	0.061 ± 0.001	0.057 ± 0.006
100 μM SNAP	8.0 ± 0.4 (132-fold)	0.60 ± 0.02 (11-fold)
200 nM PPIX	1.52 ± 0.02 (25-fold)	2.1 ± 0.4 (38-fold)
1 μM ZnPPIX	0.15 ± 0.01 (2.5-fold)	0.037 ± 0.02 (inhibition)

Note. The assay was conducted as described under Materials and Methods and was started with the addition of 0.2 or 0.5 μg of non-heme-depleted or heme-depleted enzyme, respectively, to the substrate solution containing the different porphyrins. The protein concentrations in the final reaction mixture were 6 and 16 nM, respectively, for the non-heme-depleted and heme-depleted enzyme. The porphyrin concentrations given are the final concentrations in 200-μL assay volumes. The activation is the ratio of the specific activity in the presence of the porphyrin or SNAP (100 μM) to the basal activity of sGC and is shown in parentheses. Values represent averages of two separate determinations; errors are the deviation from the mean. The dramatic decrease in activation observed after the heme-depletion treatment is indicative that the enzyme has been depleted successfully.

thermore, using metabolic labeling studies, Ingi and Ronnett (33) have demonstrated in olfactory receptor neurons a direct relationship between ^{14}CO production and endogenous cGMP concentration. Other studies showed that SnPPIX and ZnPPIX, two inhibitors of HO activity and CO production, lowered endogenous cGMP levels in peripheral neurons (32, 34) as well as in vascular endothelium (35, 36, 38). These lines of evidence suggest that CO may function as an endogenous regulator of cGMP levels; however, it remains unclear whether this effect occurs through activation of sGC since CO is such a poor activator of sGC *in vitro* (5, 21, 41–43). Importantly, a controversy exists over the selectivity of SnPPIX and ZnPPIX (35, 36, 48, 49, 51), the two HO inhibitors most frequently used in studies inferring a role for CO in neurotransmission and vasorelaxation via cGMP. As a first step toward the goal of correlating the effects of CO observed *in vitro* with those observed *in vivo* we have studied the effects of SnPPIX and ZnPPIX on sGC activity.

Interpretation of experiments where ZnPPIX and SnPPIX were used as selective HO inhibitors may be flawed because both porphyrins affect sGC activity. When ZnPPIX and SnPPIX were tested in the same concentration range used in the studies assessing the physiological role of CO (1–10 μM), ZnPPIX was predominantly an inhibitor and SnPPIX was an activator of sGC. It has been shown previously that porphyrins have a substantial effect on sGC activity. The porphyrin ring substituents, the identity of the enclosed metal ion, and the metal coordination environment affect sGC activity (18, 26, 57, 58). In fact, PPIX is known to be an activator of sGC and hydrophobic side chains at positions 2 and 4 and vicinal propionic acid residues at positions 6 and 7 of the porphyrin ring are essential for maximal enzyme activation (57). On the other hand, metalloporphyrins like FePPIX, ZnPPIX, MnPPIX, and CoPPIX have been reported to be inhibitors of sGC (25, 57). Given the known effect of a variety of nonnative porphyrins on sGC activity, it is important to investigate the effect of ZnPPIX and SnPPIX on purified sGC since it is through the use of these porphyrins that a physiological role for CO has been inferred. Our study shows that the activity of sGC is indeed affected by ZnPPIX and SnPPIX *in vitro*.

The activation by PPIX that we have observed is largely in agreement with earlier results (18, 26) and is consistent with current understanding of the mechanism of activation of sGC. Although the affinity of the protein for the porphyrin that we measure is the same within error to that of Ignarro, we never see as substantial activation by PPIX as we do with NO. The necessary, but possibly not sufficient, conditions required for a metalloporphyrin to cause sGC activation are the presence of a porphyrin ring with the correct substitution pattern (57) and the absence of a proximal bond between the protein and the metal in the porphyrin ring (25). It is now known that heme in sGC is either a five- or six-coordinate with histidine(s) as axial ligand(s) (5, 20–22). Activation of sGC by NO is initiated by binding of NO to the heme iron and proceeds

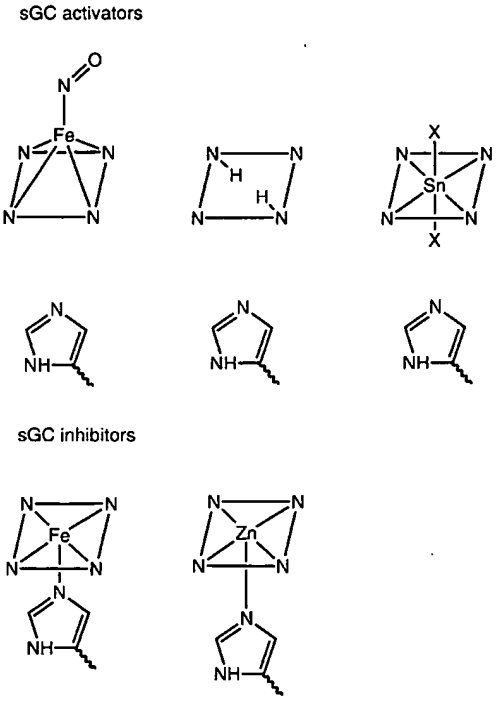

FIG. 4. Proposed coordination environments for FePPIX, FePPIX(NO), PPIX, SnPPIX, and ZnPPIX in sGC.

TABLE III

Effects of ZnPPIX and SnPPIX on HO, NOS, and sGC

Enzyme	Porphyrin	Source of the enzyme	Inhibition	Reference
HO	ZnPPIX	Human liver microsomes (HO-1)	98% inh. at 500 nM	(63)
		Spleen microsomes (HO-1)	K_i = 212 nM	(60)
		Brain microsomes (HO-2)	K_i = 3 nM	(32)
		Bovine spleen microsomes (HO-1)	K_i = 130 nM	(46)
		Rat brain extract (HO-2)	IC_{50} = 7 μM	(36)
	SnPPIX	Human liver microsomes (HO-1)	36% inh. at 2.5 μM	(63)
		Spleen microsomes (HO-1)	K_i = 11 nM	(60)
		Bovine spleen microsomes (HO-1)	K_i = 33 nM	(46)
		Rat brain extract (HO-2)	IC_{50} = 7.5 μM	(36)
sGC	ZnPPIX	Purified sGC (bovine lung)	K_i = 50 nM	(57)
		Rat cerebellar cortex (microdialysis)	55% decrease in cGMP levels at 10 μM when pretreated with SNP, no effect on basal cGMP levels	(48)
		Rat cerebellar slices	45% inh. at 100 μM	(36)
	SnPPIX	Rat cerebellar cortex (microdialysis)	55% decrease in cGMP levels at 10 μM when pretreated with SNP, no effect on basal cGMP levels; 50% reduction of basal cGMP levels at 50 μM	(48)
		Rat cerebellar slices	30% inh. at 100 mM	(36)
NOS	ZnPPIX	Cerebellum extracts	No effect at 100 μM	(48)
		Hippocampal extracts	23% inh. at 10 μM, 38% at 50 μM, and 52% at 100 μM	(49)
		Bovine aortic endothelial cells	50% inh. at 100 μM	(36)
	SnPPIX	Cerebellum extracts	No effect at 100 μM	(48)
		Bovine aortic endothelial cells	35% inh. at 100 mM	(36)

via breaking of the proximal His–Fe bond to form a five-coordinate nitrosyl–heme complex (20–22, 24, 25). In accordance with the proposed mechanism of activation, PPIX, the iron-free precursor of heme, stimulates sGC independently of NO by mimicking the conformation of the NO complex in the enzyme (Fig. 4).

SnPPIX, the most common inhibitor of HO, has a pronounced effect on sGC *in vitro*. We have demonstrated that SnPPIX is an activator of sGC with an effect on the V_{max} similar to that observed with PPIX, but a lesser effect on the affinity of the enzyme for its substrate GTP (K_m). Interestingly, sGC has a very low affinity for SnPPIX; the activation constant is in the micromolar range (K_a = 4.9 μM), whereas it is in the nanomolar range for PPIX (K_a = 11.0 nM). The poor ability of SnPPIX to compete with heme has been observed for a number of hemoproteins (59) and contrasts with its strong ability to bind heme oxygenase (K_i = 11 nM) (60). Therefore, between ZnPPIX and SnPPIX, SnPPIX is likely the most selective HO inhibitor *in vivo*.

Because of the low affinity of SnPPIX for sGC it is not possible to obtain any spectroscopic information on the interaction between SnPPIX and sGC; however, the activation observed with SnPPIX is likely to proceed by a mechanism analogous to that of PPIX and other porphyrins (25). SnPPIX used in this study was supplied as Sn(IV)PPIXCl_2 and used in aqueous solution at pH 7.4; under these conditions the chlorides are replaced by aquo ligands. We infer from the ability of SnPPIX to activate the enzyme that the six-coordinate complex, SnPPIX$(H_2O)_2^{2+}$, is bound intact to sGC (Fig. 4). The presence of two water ligands bound to the Sn(IV) ion may account for the low affinity of sGC for this porphyrin.

ZnPPIX, the second most commonly used HO inhibitor, also affects sGC activity. Our data show that ZnPPIX weakly activates sGC at concentrations up to 250 nM; above 250 nM the porphyrin becomes an inhibitor. Interestingly, a more highly heme-loaded sample of sGC was weakly activated by ZnPPIX at concentrations up to 1 μM, suggesting that the heme loading status of the enzyme plays a role in the effect observed with ZnPPIX. In contrast to SnPPIX and PPIX, ZnPPIX is a poor activator of sGC even though its activation constant resembles that of PPIX. Heme is known to be an inhibitor of sGC (57) and its replacement by a porphyrin that is less inhibitory could explain the apparent activation seen with ZnPPIX. Since sGC has a low affinity for heme (57) and heme can readily dissociate from the enzyme, sGC is usually purified as a mixture of heme-containing and heme-deficient forms (4, 61); the enzyme that we used in our experiments is such a mixture. In a previous study (57), ZnPPIX was found to be a competitive inhibitor of PPIX-activated purified sGC *in vitro* (K_i = 50 nM), revealing that ZnPPIX was binding to the same site as PPIX and with similar affinity. The ability of ZnPPIX

to replace the native Fe(II)PPIX is evidenced by the fact that the apparent affinity of sGC for ZnPPIX (K_i = 50 nM) is greater than that for Fe(II)PPIX (K_i = 350 nM) (57). Since the heme in sGC is very labile, heme exchange with a more tightly bound porphyrin is likely. Thus, the slight stimulatory effect observed could be attributed to a combination of the replacement of the preexisting heme and the reconstitution of some of the heme-deficient sGC population by ZnPPIX, resulting in an net increase in sGC activity. This hypothesis is supported by the fact that heme-depleted enzyme is only inhibited by ZnPPIX and the greater the activation of an enzyme preparation by NO, the higher the ZnPPIX concentration needed to observe inhibition of the enzyme.

Based on published information, we propose a different interaction between ZnPPIX and sGC from the one proposed for SnPPIX and PPIX. It has been shown previously that when ZnPPIX is in the presence of 2-MeImH, resonance Raman spectroscopy data are consistent with a five-coordinate ZnPPIX complex and in myoglobin, the proximal histidine coordinates to the zinc (62). It appears likely that in the case of the ZnPPIX–sGC complex, the proximal metal histidine bond is intact and the complex is five coordinate (Fig. 4). Thus, if one of the requirements necessary to obtain sGC activation is the absence of a proximal bond in between the protein and the metal in the porphyrin ring, we would not expect to observe activation with ZnPPIX. Consistent with this interpretation, only minimal activation was observed with heme-containing enzyme and inhibition is observed with heme-depleted sGC. To obtain more detailed information on the interaction between ZnPPIX and sGC, a spectroscopic study is being undertaken in our laboratory.

This study conclusively demonstrates that SnPPIX and ZnPPIX have an effect on purified sGC *in vitro* and, when considered together with other data, these observations imply that studies using metalloporphyrins as inhibitors of HO must be carried out and interpreted with care. Table 3 summarizes a wide variety of data reflecting the effect of SnPPIX and ZnPPIX on the activities of HO, sGC and NOS. In studies supporting a physiological role for CO through activation of sGC, concentrations of HO inhibitors in the range of 1–10 μM are commonly used to demonstrate involvement of HO-derived CO in the response. At these concentrations, inhibition of NOS by ZnPPIX and SnPPIX is minimal, as illustrated by the data in Table 3, but Luo and Vincent (1994) (48) showed that in the same concentration range (1–10 μM), ZnPPIX and SnPPIX are significant inhibitors of NO-dependent cGMP production *in vivo* when applied to the rat cerebellar cortex through a microdialysis probe. We have demonstrated that when ZnPPIX was tested at 2.5 μM, it inhibited the basal activity of purified sGC by about 80%, whereas 2.5 μM SnPPIX activated the enzyme. The effect of these porphyrins appears to be modulated by the heme content of the enzyme, as evidenced by our data demonstrating that sGC responds differently to SnPPIX and ZnPPIX depending on the heme-loading status of the enzyme, and by the relative concentrations of porphyrin and protein, as evidenced by the variability in the response of the enzymes of the NO–cGMP pathway (HO, sGC, NOS) to SnPPIX and ZnPPIX in the differing studies reported in Table 3. Consequently, the effect of metalloporphyrins on the activity of sGC may be difficult to predict based on concentration alone. Based on the available data, it appears that in the nanomolar concentration range, SnPPIX is likely to be the most selective inhibitor of HO since sGC has a low affinity for this metalloporphyrin.

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Photodynamic therapy with systemic administration of photosensitizers in dermatology

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Abstract

We have reviewed the results of clinical investigations into the use of photodynamic therapy (PDT) with intravenous injection of hematoporphyrin derivative (HpD), Photofrin (PF) and Sn-protoporphyrin (Sn-Pp) or oral administration of δ-aminolevulinic acid in the treatment of skin cancers and/or psoriasis. Bowen's disease was highly responsive, provided that adequate light and HpD or PF doses were delivered. In contrast, poor results were shown for squamous cell carcinoma, and the rates of complete response of basal cell carcinoma ranged between 0% and 100%. Treatment failures could be related to the delivery of low drug and/or light doses, but differences in the thickness and pigmentation of the treated lesions may play a relevant role. Good palliation was almost always achieved in patients affected by primary and secondary breast carcinomas, although complete eradication of tumors was very rare. PDT is a very promising treatment modality for both Mediterranean and HIV-related Kaposi's sarcoma, because it appears to be effective, can be repeated and is not associated with immunosuppressive activity or significant systemic toxicity. PDT of psoriasis with low doses of Sn-Pp, HpD or PF plus UVA light and PF plus 630 nm light proved to be effective and was associated with mild, dose-related and reversible photosensitivity.

Keywords: Breast carcinoma; Hematoporphyrin derivative; Kaposi's sarcoma; Photodynamic therapy; Photofrin; Psoriasis; Skin cancer

1. Introduction

Photodynamic therapy (PDT) is based on the activation of a photosensitizing and tumor-localizing dye with specific wavelengths of non-ionizing electromagnetic radiation. Photochemical reactions lead to cell damage, mainly via the production of singlet oxygen, although other radical species (perhaps superoxide) cannot be ruled out.

Hematoporphyrin derivative (HpD), Photofrin (PF) and tin-protoporphyrin (Sn-Pp) are first-generation PDT photosensitizers, and the only agents for which extensive clinical data are available on the treatment of skin cancers and/or psoriasis. A single intravenous bolus push is the standard method of delivery of these drugs. Irradiation of the tumor area is most frequently performed 48–72 h after drug administration, because at this time the ratio of photosensitizer concentration between tumor and peritumoral tissues is particularly large [1]. However, normal skin may retain detectable amounts of porphyrins for several weeks and prolonged skin photosensitivity is the major adverse side-effect of the therapy.

Intravenously delivered benzoporphyrin derivative monoacid ring A (BPD), mono-*l*-aspartyl chlorin e6 (NPe6) and chloro-aluminum sulfonated phthalocyanines (CASPc) and orally administered δ-aminolevulinic acid (ALA), a precursor of protoporphyrin IX in the metabolic pathway to heme, are currently under investigation in order to enhance the efficacy of the treatment and reduce the average duration of photosensitivity.

The present review is focused on the clinical results of PDT in the treatment of skin cancers and psoriasis, but we state in advance that this task is very challenging because the studies involved uncontrolled open trials on small numbers of patients and anecdotal reports with wide variation of drug and light doses, duration of follow-up as well as criteria of tumor and patient selection. In addition, the results are difficult to compare because they were reported as the number of complete or partial responses with respect to the number of treated patients, lesions, sites, treatment sessions or courses. Finally, grading of the responses was almost always made on the basis of the pure clinical criterion of macroscopic tumor disappearance at the end of therapy, and responses were con-

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sidered "complete" although they recurred at follow-up or persistent tumor cells were seen at histologic examination.

2. Clinical uses of systemic PDT in dermatology

2.1. Non-melanoma skin cancers

A large number of clinical studies on the PDT of non-melanoma skin cancers (NMSCs), i.e. Bowen's disease (BD), basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), have been carried out.

PDT was very effective in the treatment of BD provided that sufficient combinations of drug and light dose were delivered. PF (2.0 mg kg^{-1}), followed by irradiations of 25 J cm^{-2} [2], 50 J cm^{-2} [3] and 20–40 J cm^{-2} [4] of 630 nm light, was effective in treating 500/500 (100%) and 49/50 (98%) treated lesions and 3/3 (100%) irradiated sites respectively. Half doses of PF (1.0 mg kg^{-1}) were effective in all eight lesions irradiated with 185–250 J cm^{-2} of 630 nm light [5], but only 50% of the lesions responded if 50 J cm^{-2} of 628 nm light [2] or 50–100 J cm^{-2} of 630 nm light [3] were delivered.

McCaughan et al. [6] reported that one of two treatment sessions delivered to a patient affected by BD was followed by a recurrence after 1 year. However, it is unclear how many lesions were treated and if HpD (3.0 mg kg^{-1}) or PF (2.0 mg kg^{-1}) was used (Table 1).

SCCs were poorly responsive to PDT in two investigations. A total of 32 SCCs were apparently eradicated by treatment with 5.0 mg kg^{-1} HpD, followed by irradiation with 30 J cm^{-2} of red light, but more than half recurred within 6 months [7]. Three patients were treated with either HpD (3.0 mg kg^{-1}) or PF (2.0 mg kg^{-1}) and 20–30 J cm^{-2}, but only two of the five treatment sessions, where more lesions could be treated at the same time, were effective [6]. Unfortunately, tumor thickness was not reported in these studies [6,7]. In an anecdotal case, 2.0 mg kg^{-1} PF and 630 nm light irradiation at a dosage of 150 J cm^{-2} cured a large superficial SCC of the lower lip without recurrence after more than 6 months [8] (Table 1).

The PDT of BCCs has been investigated in a number of studies with contrasting results. The first worker to report the complete disappearance of a basalioid tumor with PDT was Dougherty [9] in 1981. He performed the treatment with 5.0 mg kg^{-1} HpD and two daily irradiations of 120 J cm^{-2} of 600–700 nm light from a filtered xenon arc lamp. In addition, he mentioned the successful treatment of four other BCCs by his group [9]. Afterwards, other excellent results were reported: 33 (89.2%) complete responses out of 37 lesions of three patients affected by basal cell nevus syndrome who received 3.0 mg kg^{-1} HpD plus 38–180 J cm^{-2} of either 600–700 nm or 630 nm light [10]; 100% complete responses of three sites treated with 1.5–2.0 mg kg^{-1} PF and 40–60 J cm^{-2} [4]; 14 complete responses (93.3%) out of 15 lesions treated with 2.0 mg kg^{-1} PF and 50 J cm^{-2} [2]; 134 out of 151 (88.7%) lesions treated with 1.0 mg kg^{-1} PF and 72–288 J cm^{-2} [11]. The last study reported the longest follow-up of 20–43 months.

Calzavara and Tomio [12] observed high cure rates after the treatment of 17 lesions with either Hp (5.0 mg kg^{-1}) or HpD (2.5–3.0 mg kg^{-1}) plus 25–225 J cm^{-2} of light from a filtered xenon arc lamp. However, recurrences were observed within 6 months and the final rate was 58.8%. Bandieramonte et al. [13] observed 25/42 (59.5%) complete responses 60 days after treatment with 3.0 mg kg^{-1} HpD and 60–120 J cm^{-2} from argon ion or dye lasers without recurrence after 4–16 months.

Three papers reported poor results. Buchanan et al. [3] found that 1.5–2.0 mg kg^{-1} PF plus 50–100 J cm^{-2} were effective only in 5/13 (38.1%) patients with single or multiple BCCs. McCaughan et al. [6] observed only four (14.8%) complete responses after 12 months after the delivery of 27 courses with either PF (2.0 mg kg^{-1}) or HpD (5.0 mg kg^{-1}) and 20–30 J cm^{-2}. Finally, all 21 BCCs, treated by Pennington et al. [7] with 5.0 mg kg^{-1} HpD and 30 J cm^{-2}, recurred 6 months later (Table 1). The different results in the treatment of NMSCs may be explained by differences in drug and light doses, differences in thickness and pigmentation of the treated lesions and differences in sensitivity to the treatment and biological behavior of the various types of NMSC.

Clinical evidence seems to indicate that the PDT of NMSCs is effective if adequate combinations of drug and light dose are delivered. Robinson et al. [2] and Buchanan et al. [3] demonstrated that there is no simple reciprocity between the drug and light dose for obtaining the same therapeutic effect [14]. They found a 50% reduction of the efficacy of PDT of BD by carrying out treatment with half the drug dose and twice the light dose. On the whole, the worst results in the treatment of any type of NMSC were obtained by Pennington et al. [7] and McCaughan et al. [6] who delivered full doses of PF followed by doses of light equal to or lower than 20–30 J cm^{-2}.

Differences in the depth of invasion may be another critical factor, because deep dermal or subcutaneous projections may be inadequately reached by light. Bandieramonte et al. [13] found that the therapeutic effect for BCCs is dependent on the tumor infiltration patterns, and that BD (intraepidermal, superficial or mildly elevated lesions) is more responsive than SCCs and BCCs whose clinical spectra vary from superficial to deeply invading and elevated lesions. Other morphological characteristics, such as heavy pigmentation of pigmented BCCs and fibrous stroma of morpheaform BCCs, represent additional obstacles to the therapy [11].

2.2. Primary and metastatic breast carcinoma

PDT has been used for the treatment of primary and locally recurrent breast carcinoma. Almost all patients had failed one or more conventional therapies (radiation therapy, chemotherapy, hormonal therapy, surgical resection) or were con-

Table 1
Systemic photodynamic therapy of non-melanoma skin cancers

Reference	Drug (mg kg^{-1})	Light (nm)	Light dose (J cm^{-2})	Pts	Complete responses [a] (%)	Follow-up (months)
Bowen's disease						
Waldow et al. [4]	PF (2.0)	630	40–60	2	3/3 [b] (100%)	3
Robinson et al. [2]	PF (2.0)	628	25	2	>500/>500 [c] (100%)	6
	PF (1.0)	628	50	1	45/90 [c] (50%)	1
Buchanan et al. [3]	PF (2.0)	630	50	1	49/50 [c] (98%)	ND
	PF (1.0)	630	50–100	1	ND/ND [c] (50%)	ND
McCaughan et al. [6]	HpD (3.0) PF (2.0)	630	20–30	1	1/2 [d] (50.0%)	12
Jones et al. [5]	PF (1.0)	630	185–250	6	8/8 [c] (100%)	14–24
Squamous cell carcinoma						
Pennington et al. [7]	HpD (5.0)	630	30	6?	ND/32 [c] (<50%)	6
McCaughan et al. [6]	HpD (3.0) PF (2.0)	630	20–30	3	2/5 [d] (40.0%)	12
Gross et al. [8]	PF (2.0)	630	150	1	1/1 [c] (100%)	6
Basal cell carcinoma						
Dougherty [9]	HpD (5.0)	600–700	2×120	1	1/1 [c] (100%)	7
Tse et al. [10]	HpD (3.0)	600–700 630	38–180	3	33/40 [c] (82.5%)	12–14
Bandieramonte et al. [13]	HpD (3.0)	480–515 630	60–120	4	25/42 [c] (59.5%)	4–16
Waldow et al. [4]	PF (1.5–2.0)	630	40–60	4	6/6 [b] (100%)	3
Pennington et al. [7]	HpD (5.0)	630	30	6?	0/21 [c] (0%)	6
Robinson et al. [2]	PF (2.0)	628	50	1	14/15 [c] (93.3%)	6
Buchanan et al. [3]	PF (1.5–2.0)	630	50–100	13	5/13 [c] (38.5%)	ND
McCaughan et al. [6]	HpD (3.0) PF (2.0)	630	20–30	7	4/27 [d] (14.8%)	12
Calzavara and Tomio [12]	Hp (5.0) HpD (2.5–3.0)	600–700	25–225	ND	10/17 [c] (58.8%)	>6
Wilson et al. [11]	PF (1.0)	630	72–288	37	134/151 [c] (88.7%)	20–43

[a] Macroscopic disappearance of the lesion at the end of therapy without recurrence at follow-up or persistence of tumor remnants at histological examination.
[b] Sites.
[c] Lesions.
[d] Sessions.
[e] Patients.

sidered unsuitable for these therapies because of their bad general condition. The aim was to eradicate or, at least, to reduce the nodules in order to prevent their coalescence into ulcerated masses. Therefore both "complete" and "partial" responses may be considered as positive results. In addition, the degree of response was largely approximate, because it was assessed without serial histologic examination of the whole treated area and without prolonged follow-up, as metastases were present in other body sites and the patients did not survive long enough (Table 2).

The overall percentage of complete plus partial responses was 97.1% in 35 patients treated with 2.5–5.0 mg kg^{-1} HpD by Dougherty [9]. Injection of 3.0 mg kg^{-1} HpD or 2.0 mg kg^{-1} PF and irradiation with 60–120 J cm^{-2} resulted in complete (21) or partial (8) tumor responses in the study of McCaughan et al. [6]. Carruth and McKenzie [15] treated five patients with nine courses of 3.0 mg kg^{-1} HpD and 25 J cm^{-2} and obtained four complete and four partial responses. The only patient treated with 1.5 mg kg^{-1} PF and the same light dose showed a complete response [15]. Afterwards, the same group reported that complete or partial responses were always obtained in seven patients treated with 3.0–4.0 mg kg^{-1} HpD and eight patients treated with 1.5–2.0 mg kg^{-1} PF followed by irradiation with 630 nm laser light at a dosage of 25–200 J cm^{-2} [3]. However, in both studies, the follow-up was very short for most patients [3,15].

Schuh et al. [16] treated 14 patients with 30 PDT courses of PF at doses in the range 1.0–2.0 mg kg^{-1} followed by irradiation with 36–288 J cm^{-2} of 630 nm laser light. In addition, three patients received 100–440 J cm^{-2} of light through diffusers implanted interstitially; 24 (80%) courses yielded complete or partial responses.

Sperduto et al. [17] found that 13 out of 20 (65%) patients achieved complete or partial response after receiving 1.5 mg kg^{-1} PF and 20–359 J cm^{-2} of 630 nm laser light.

Waldow et al. [4] treated successfully 30/38 (78.9%) sites of one patient with 3.0 mg kg^{-1} HpD and 8–11 J cm^{-2} and 108/108 (100%) sites of three patients with 2.0 mg kg^{-1} PF and 20–60 J cm^{-2}. These results were confirmed by biopsy no sooner than 3 months after the treatment.

Table 2
Primary and metastatic breast carcinoma

Reference	Drug (mg kg^{-1})	630 nm light (J cm^{-2})	Pts	Responses (complete + partial) [a] (%)	Recurrences/followed-up lesions	Follow-up (months)
Dougherty [9]	HpD (2.5–5.0)	?	35	34/35 [b] (97.1%)	6/16	2–12
Bandieramonte et al. [13]	HpD (3.0)	60–120	2	9/18 [c] (50.0%)	ND	8–16
Carruth and McKenzie [15]	HpD (3.0)	25	5	8 (4 + 4)/9 [d] (88.9%)	ND	ND
	PF (1.5)	25	1	1 (1 + 0)/1 [d] (100%)	0/1	4
Waldow et al. [4]	HpD (3.0)	8–11	1	30 (15 + 15)/38 [e] (78.9%)	0/30	3
	PF (2.0)	20–60	3	108 (93 + 15)/108 [e] (100%)	0/108	3
Schuh et al. [16]	PF (1.0–2.0)	36–288	14	24 (2 + 22)/30 [d] (80%)	24/24	1.5–8
McCaughan et al. [6]	HpD (3.0) PF (2.0)	20–30	12	29 (21 + 8)/29 [f] (100%)	ND	12
Buchanan et al. [3]	HpD (3.0–4.0)	25–200	7	7 (2 + 5)/7 [c] (100%)	ND	ND
	PF (1.5–2.0)	25–200	8	8 (1 + 7)/8 [c] (100%)	ND	ND
Sperduto et al. [17]	PF (1.5)	20–359	20	13 (4 + 9)/20 [b] (65%)	13/13	1.5–14

[a] Clinical evaluation at 30 or 60 days after the end of treatment.
[b] Patients.
[c] Lesions.
[d] Courses.
[e] Sites.
[f] Sessions.

Apparently, the worst results (9/18 treated sites) were obtained by Bandieramonte et al. [13] with the delivery of 3.0 mg kg^{-1} HpD and irradiation with 60–120 J cm^{-2}. However, these results were assessed by pathologic examination of multiple biopsy samples from treated sites 2 months after treatment.

Differences in treatment results are not apparently related to the delivery of combinations of low doses of drug and/or light. However, in one study, the average total dose was proportional to the degree of responsivity: 140 J cm^{-2} to complete responders, 61 J cm^{-2} to partial responders and 46 J cm^{-2} to the no-responder group [17].

The major factor limiting the irradiation of high doses of light to lesions below the skin is the risk of photoactivation of the photosensitizer in the overlying and adjacent normal skin [9]. Overtreatment may cause large eschars that are quite painful to the patient and may require 1–2 months to lift leaving some contracture and scarring [3]. However, there is no complete agreement on the light–drug combinations that can be safely delivered for the treatment of subcutaneous tumors with the preservation of the overlying and adjacent skin. The upper limits of the light dose were 25–35 J cm^{-2} for 3.0 mg kg^{-1} HpD and 35–50 J cm^{-2} for 2.0 mg kg^{-1} PF according to Dougherty [18], 20–30 J cm^{-2} in Caucasians and 40 J cm^{-2} in blacks with 3.0 mg kg^{-1} HpD or 2.0 mg kg^{-1} PF according to McCaughan [19] and 25 J cm^{-2} following injection of 3.0–4.0 mg kg^{-1} HpD or 1.5–2.0 mg kg^{-1} PF according to Buchanan et al. [3]. Schuh et al. [16] and Mang et al. [20] suggested 36 J cm^{-2} with 2.0 mg kg^{-1} PF, but Sperduto et al. [17] reported that 35 J cm^{-2} following the injection of 1.5 mg kg^{-1} PF caused full thickness chest wall necrosis.

The outcome of the therapy can also be influenced by the extent and thickness of the lesions, as thick and bulky lesions appear to be more resistant to PDT [10,12,13,16].

Lesions with a thickness of 1 cm or more can theoretically be treated provided that very high fluences are delivered with exposure times that are prohibitively long. Therefore it is better to debulk the large tumors and treat the remaining tissue bed with PDT [6] or to use interstitial diffusers of 1–3 cm in length [6,9,18]. Diffusers allow a radius of necrosis of 5–10 mm, and therefore tumors larger than this should receive multiple treatment, preferably simultaneously [18]. Therapeutic efficacy is not influenced by the histologic type of the carcinoma [16], but diffuse inflammatory metastates are poorly responsive and the treatment is associated with extensive cutaneous necrosis and pain.

2.5. *Kaposi's sarcoma*

Dougherty [9] with 2.5 mg kg^{-1} HpD plus 120 J cm^{-2} and Calzavara and Tomio [12] with 2.5–3.0 mg kg^{-1} HpD plus 50–200 J cm^{-2} obtained the clinical disappearance of 100% treated sites in three patients and 85% treated sites in four patients respectively affected by classic Mediterranean Kaposi's sarcoma (KS). Schweitzer and Visscher [21] treated five patients affected by aggressive KS associated with AIDS with the administration of 2.0 mg kg^{-1} PF and surface or interstitial delivery of 50–200 J cm^{-2}. Total disappearance or flattening of the cutaneous and mucosal nodular lesions without growth for at least 8 weeks was obtained in 54/92 (58.7%) treated sites.

One of us (P.G.C.) treated three patients affected by classic KS with oral administration of 60 mg of ALA. ALA is

not a photosensitizer per se, but it is the precursor of photosensitive protoporphyrin IX in the biosynthetic pathway to heme. Lesions showed a good fluorescence, but after irradiation with 50–100 J cm^{-2} of 630 nm light, a reduction of less than 30% was observed. At histologic examination, wedges of necrotic tumor tissue were observed 6 mm from the skin surface and were intermingled with persistent tumor areas.

2.4. *Cancers of dermatological interest*

Although several people have attempted to treat metastases of pigmented melanoma using PDT, little of this is reflected in the literature [9]. Dougherty [9] reported that treatment with 3.75 mg kg^{-1} HpD and 60–140 J cm^{-2} of 630 nm light resulted in the eradication of more than 100 melanoma metastases. Three patients affected by malignant melanoma were treated by McCaughan et al. [6] with 3.0 mg kg^{-1} HpD or 2.0 mg kg^{-1} PF and doses of 630 nm laser light ranging between 20 and 30 J cm^{-2}. Five of the seven treatment sessions were followed by complete responses, but only one patient was free of recurrence after 14 months [6]. A partial response was achieved in an amelanotic melanoma treated with 3.0–4.0 mg kg^{-1} HpD and 100–200 J cm^{-2} of 630 nm laser light [3]. Metastatic amelanotic melanoma and malignant fibrous hystiocytoma failed to respond to 3.0 mg kg^{-1} HpD and 100–200 J cm^{-2} of 630 nm light in the study of Carruth and McKenzie [15]. A patient affected by liposarcoma was still free of recurrence 12 months after surgical ablation and PDT of the surgical tumor bed [6]. Eradication of a capillary hemangioma with HpD and 11 J cm^{-2} of 630 nm coherent light was reported by Keller et al. [22] with no injury of the overlying skin.

2.5. *Psoriasis*

Silver [23] reported the clinical use of hematoporphyrin and UV light in the treatment of psoriasis as early as 1937.

Berns et al. [24] treated a patient with vulvar neoplasia who had coexisting psoriasis using HpD (3.0 mg kg^{-1}). The psoriasis treatment area was divided into two regions receiving 20 and 40 J cm^{-2} of 630 nm laser light. Both areas formed eschars by 1 week post-irradiation and underwent normal re-epithelization within 17 days. Eschar formation suggested tissue destruction at a greater depth than necessary.

Low doses of HpD (1.0 mg kg^{-1}) and the daily whole body delivery of UVA light for 15 days was a safe and effective treatment of widespread plaque-type psoriasis, because an improvement greater than 90% was achieved in 15 out of 19 patients without significant side-effects [25].

Repetitive daily UVA irradiations were also used by Emtestam et al. [26] in the treatment of 10 psoriatic patients after a single injection of 2.0 μmol kg^{-1} of Sn-Pp. Sn-Pp is a synthetic heme analog which inhibits heme oxygenase, the rate-limiting enzyme in the catabolism of heme to bilirubin. The light sensitivity induced by Sn-Pp resulted in a slightly increased erythemal and tanning reaction to sunlight in most patients. This effect lasted for up to 2 months after the injections. The cumulative UVA dose was 98.3 ± 35.1 J cm^{-2} fractionated in 21 days. Psoriatic lesions improved in all patients and the mean clinical score fell from 7.9 to 3.6.

The use of UVA light by Berg et al. [25] and Emtestam et al. [26] seems to indicate that the photoactivation of the drug by red light is not necessary for obtaining the therapeutic effect for psoriasis.

However, the usefulness of different absorption bands of porphyrins is still debated. Weinstein et al. [27] delivered a single intravenous dose of 0.5 mg kg^{-1} PF to eight patients, and exposed different sites of lesional skin to various dose schedules of 630 nm, 405 nm or UVA light. The results suggest that PDT with PF and 405 nm or UVA irradiation is not clinically useful, whereas treatment with red light provides a safe and useful alternative therapy for psoriasis.

3. Side-effects

PDT is slightly or not painful and general anesthesia was requested only for treating oral KS in HIV-positive patients [21]. The cosmetic and functional outcome is superior to other treatment modalities in many clinical situations. However, if overtreatment is delivered, patients experience moderate to severe pain [3] when the tumor sloughs and the area becomes covered with a dark eschar which separates leaving scars and contractures.

Intravenously delivered dyes are uniformly distributed to the entire skin surface. Clinical photosensitivity usually persists for 6–8 weeks and is dose related [28]. However, good compliance was obtained by almost all patients. Cases of mild to severe sunburn of exposed areas were seldom reported and healed with anti-inflammatory treatment [9].

PDT is not associated with liver, kidney or other internal toxicity, except a transitory 30% decrease in bilirubin levels after Sn-Pp injection [26]. However, patients with severe liver or kidney disease should avoid the treatment, because porphyrins are retained longer in the liver, spleen and kidneys.

4. Conclusions and perspectives

The review of clinical studies suggests that PDT is effective in the treatment of selected cases of skin tumors, and prolonged photosensitization is the only relevant side-effect.

Systemic PDT with adequate combinations of light and drug doses appears to be as effective as other standard therapeutic modalities in the treatment of BD. The risk of sunburn could be minimized for these patients by delaying PDT until the winter months, because only a small fraction (5%) of BD may evolve to SCCs and this evolution is usually very slow [29].

In contrast, the reported cure rates of SCCs are lower than those of surgery, Mohs' surgery and radiotherapy [30]. Its

use should be avoided for nodular lesions that have a high risk of metastatization and are presumably less responsive.

Results of PDT in the treatment of BCCs are contrasting. In a few investigations, PDT was not (or poorly) effective [3,6,7], but in others it approached or was superior to the more than 90% cure rates [2,4,9–11] obtained with standard therapies [31]. A careful selection of cases, with the exclusion of pigmented, morpheaform and thick nodular lesions, could probably further enhance the cure rates.

At present, PDT of NMSCs with intravenous delivery of HpD or PF seems to be particularly valuable for three small subgroups of patients: patients with multiple NMSCs, particularly patients affected by basal cell nevus syndrome or chronic arsenicism, patients with large and superficial NMSCs in areas in which surgery would be cosmetically disfiguring, and seriously ill patients with large NMSCs who are unfit to undergo surgery with general anesthesia. However, for the great majority of clinical situations, prolonged photosensitivity is a serious drawback of the therapy and further evidence from controlled studies is needed for a definite assessment of its efficacy. Systemic PDT appears to be effective for the palliation of primary and metastatic breast carcinoma, but guidelines for patient selection and treatment guidelines are lacking.

Preliminary experience in the therapy of advanced classic and AIDS-related KS seems to indicate that PDT may become preferable to standard therapies, i.e. ionizing radiation and mono- or poly-chemotherapy, because it is very effective and may be repeated several times without causing cumulative side-effects. Although systemic PDT in certain mouse models with the irradiation of large areas has caused immunosuppression [32,33], no indication for such an effect exists in humans.

In the therapy of psoriasis, the major potential advantage of PDT over psoralen plus UVA (PUVA) treatment is that the photodynamic reactions involving porphyrins do not appear to be carcinogenic. However, the treatment is unsuitable for most patients because of prolonged photosensitivity.

Progress is being made in the study of new second-generation photosensitizers, but phase II/III trials with BPD [34] and phase I/II trials with NPe6 and CASPc [28] are still ongoing.

Levy et al. [34] have recently anticipated that over 50 patients with a variety of NMSCs have been treated with 0.3 mg kg^{-1} body weight of liposomal BPD and 690 nm laser light 2–6 h after injection. The complete response rate was approximately 100%. In another experimental trial, multiple 2 cm^2 portions of psoriatic skin were treated with 25, 50 and 75 J cm^{-2} of 690 nm light after intravenous injection of BPD. BPD at a dosage of 0.2 mg kg^{-1} and the highest light dose achieved clearing of psoriatic lesions for periods of at least 60 days [34].

Systemic administration of 30–60 mg kg^{-1} ALA to four patients affected by advanced squamous cell carcinoma of the mouth allowed the accumulation of protoporphyrin IX in tumor cells 3 h later, returning to background in 24 h. The tumor fluorescence intensity was about twice that of the surrounding connective tissues. Irradiation with 50–100 J cm^{-2} laser light at 630 nm caused palliative tumor necrosis in three patients [35]. Treatment was associated with an increase in serum aspartate aminotransferase and bilirubin levels. However, with the same treatment protocol, we did not obtain good palliation in three patients affected by classic KS.

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